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Unraveling the relationship between macula densa cell volume and luminal solute concentration/osmolality

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At the macula densa, flow-dependent changes in luminal composition lead to tubuloglomerular feedback and renin release. Apical entry of sodium chloride in both macula densa and cortical thick ascending limb (cTAL) cells occurs via furosemide-sensitive sodium–chloride–potassium cotransport. In macula densa, apical entry of sodium chloride leads to changes in cell volume, although there are conflicting data regarding the directional change in macula densa cell volume with increases in luminal sodium chloride concentration. To further assess volume changes in macula densa cells, cTAL-glomerular preparations were isolated and perfused from rabbits, and macula densa cells were loaded with fluorescent dyes calcein and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate. Cell volume was determined with wide-field and multiphoton fluorescence microscopy. Increases in luminal sodium chloride concentration from 0 to 80 mmol/l at constant osmolality led to cell swelling in macula densa and cTAL cells, an effect that was blocked by luminal application of furosemide. However, increases in luminal sodium chloride concentration from 0 to 80 mmol/l with concomitant increases in osmolality caused sustained decreases in macula densa cell volume but transient increases in cTAL cell volume. Increases in luminal osmolality with urea also resulted in macula densa cell shrinkage. These studies suggest that, under physiologically relevant conditions of concurrent increases in luminal sodium chloride concentration and osmolality, there is macula densa cell shrinkage, which may play a role in the macula densa cell signaling process.

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With increased luminal flow in the loop of Henle, macula densa cell activation occurs in response to a concomitant elevation in luminal fluid sodium chloride concentration ($[NaCl]_L$) and osmolality (osm_L).[1,2] This results in paracrine signaling to the afferent arteriole to adjust preglomerular vascular tone (a phenomenon called tubuloglomerular feedback (TGF) response) and to contribute to the regulation of renin release from the granular cells. The alterations in $[NaCl]_L$ at the macula densa segment are thought to occur between ~15 and 60 mmol/l, with concomitant changes in osm_L between ~100 and 150–200 mOsm/kg H_2O.[3] Early studies suggested that macula densa cells transport NaCl via a furosemide-sensitive $Na^+:2Cl^-:K^+$ cotransporter, and that these cells exhibited at least some degree of apical water permeability.[4–7] The finding that macula densa cells might be a water-permeable 'window' in the otherwise water-impermeant cortical thick ascending limb (cTAL) was the base of observations by Kirk *et al.*,[8] using differential interference contrast microscopy. It was found that parallel increases in $[NaCl]_L$ and osm_L from 26 to 146 mmol/l and from 70 to 290 mOsm/kg H_2O, respectively, led to reversible decreases in macula densa cell height and narrowing of the lateral intercellular spaces. Also, transmission electron microscopic studies demonstrated closure of intercellular spaces between macula densa cells in cases where $[NaCl]_L$ and osm_L at the macula densa were expected to rise.[9] According to both studies, changes in lateral intercellular spaces and cell height were specific to the macula densa cells and there were no changes in the surrounding cTAL. Studies by Gonzalez *et al.*[10,11] also suggested that parallel increases in $[NaCl]_L$ and osm_L lead to decreases in macula densa cell height. Thus, these studies indicate that concordant elevations in $[NaCl]_L$ and osm_L lead to cell shrinkage owing to a change in the osmotic gradient across the apical membrane.

In contrast, others reported $[NaCl]_L$ elevation-induced increases in macula densa cell volume, using fluorescence microscopy.[12,13] Also, a study from our laboratory concluded that elevations in $[NaCl]_L$ result in macula densa cell swelling.[14] However, these studies were performed by altering $[NaCl]_L$ while maintaining osm_L constant or minimally altered.[14] Thus, there appears to be a conundrum regarding the effect of increasing $[NaCl]_L$ on macula densa cell volume.

The importance of a detailed knowledge of macula densa cell volume regulation is best demonstrated by speculations that cell swelling might play a role in adenosine 5′ triphosphate release from macula densa cells induced by changes in $[NaCl]_L$,[15,16] and that macula densa cell shrinkage would participate in the signaling of $[NaCl]_L$-dependent prostaglandin E_2 release from macula densa cells.[17,18] The present studies were performed to re-evaluate changes in macula densa cell and cTAL epithelial cell volume upon alterations in $[NaCl]_L$ at constant or varying osm_L and upon changes in luminal urea concentration.

RESULTS

Effect of increases in $[NaCl]_L$ on macula densa cell volume as assessed by cytosolic dye concentration

One approach to determine changes in cell volume is to use the fluorescent probe calcein, which can be loaded into cells as an ester form and is trapped inside the cells upon cleavage of the methyl ester. Unlike fura-2 or other ionic probes, this dye is not sensitive to changes in intracellular ionic composition. However, when cells swell or shrink, changes in calcein dye concentration can be used as an index of alterations in cell volume. For example, an increase in cell volume is expected to result in a dilution of the dye and a decline in fluorescence. Studies were performed to compare cell volume responses in macula densa versus adjacent cTAL cells (Figure 1). Increases in osm_L caused increases in calcein fluorescence, whereas decreases in osm_L led to decreases in intensity, indicating an inverse relationship between cell volume and calcein fluorescence intensity. As shown in Figure 2a, increases in $[NaCl]_L$ from 0 to 80 mmol/l at constant osm_L of 210 mOsm/kg H_2O produced reversible, dose-dependent decreases in calcein intensity in the macula densa cells, suggesting reversible cell swelling. Luminal application of 10^{-4} mol/l furosemide, inhibitor of Na^+:$2Cl^-$:K^+ cotransporter, reduced the magnitude of changes in calcein intensity upon increases in $[NaCl]_L$ at constant osm_L by $83 \pm 11\%$ ($n = 6$; $P < 0.05$). In contrast, parallel increases n $[NaCl]_L$ and osm_L from 0 to 80 mmol/l and from 60 to 210 mOsm/kg H_2O (Figure 2b) caused increases in calcein fluorescence, indicating cell shrinkage. As shown in Figure 2c, concomitant increases in $[NaCl]_L$ and osm_L produced dose-dependent decreases in macula densa cell volume. The comparison of volume responses to changes in luminal fluid composition between macula densa and cTAL produced strikingly different responses. As shown in Figure 3a, both cell types produced cell swelling in response to increases in $[NaCl]_L$ at constant osm_L. In contrast, increases in both $[NaCl]_L$ and osm_L produced cell shrinkage in macula densa cells and cell volume increase in cTAL cells. Interestingly, cell volume responses in macula densa cells were sustained, whereas the volume responses in cTAL were

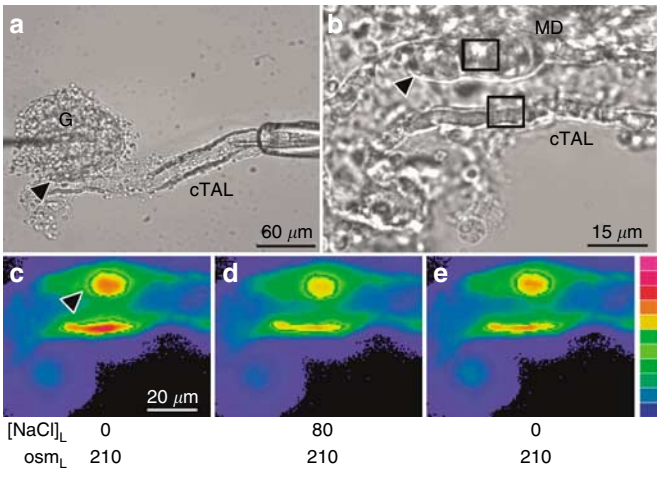

Figure 1 | Imaging of cell volume changes in the isolated perfused cTAL-glomerulus (G) using the cytosolic dye calcein. (**a**, **b**) Low-power and high-power bright-field images of the preparation. The squares represent regions of interest windows in macula densa (MD) and cTAL cells. (**c**–**e**) Wide-field fluorescence images demonstrating the effect of alterations in $[NaCl]_L$ (shown in mmol/l below the images) at constant osm_L (indicated in mOsm/kg H_2O) on calcein fluorescence intensity. The color scale spans from 0 to 2816 intensity units. Arrowhead denotes macula densa plaque.

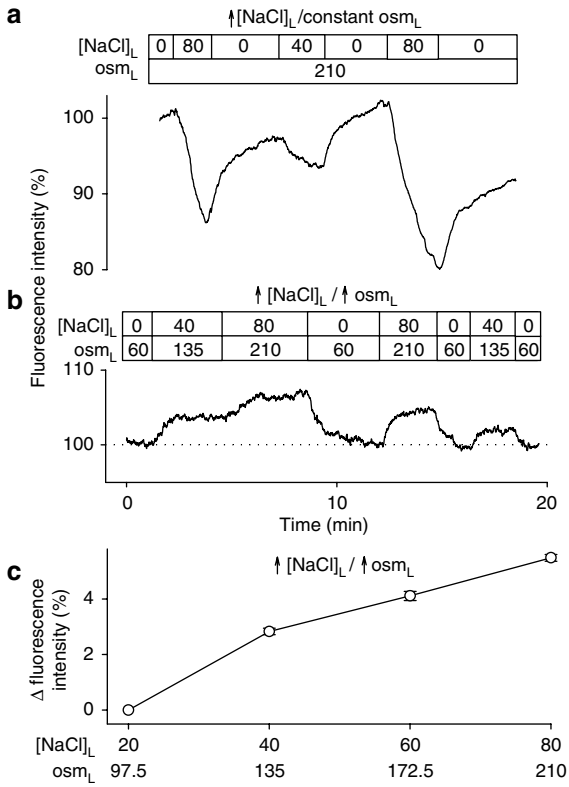

Figure 2 | Effect of $[NaCl]_L$ and osm_L on macula densa cell volume. Representative tracings of calcein fluorescence intensity recorded from macula densa plaques upon changes in $[NaCl]_L$ (shown in mmol/l above the tracings) (**a**) at constant osm_L (in mOsm/kg H_2O) and (**b**) with concomitant changes in osm_L. (**c**) Dose–response relationship between $[NaCl]_L$ and calcein fluorescence intensity in macula densa cells upon concomitant alterations in osm_L ($n = 4$–9; all values are different from each other, $P < 0.05$).

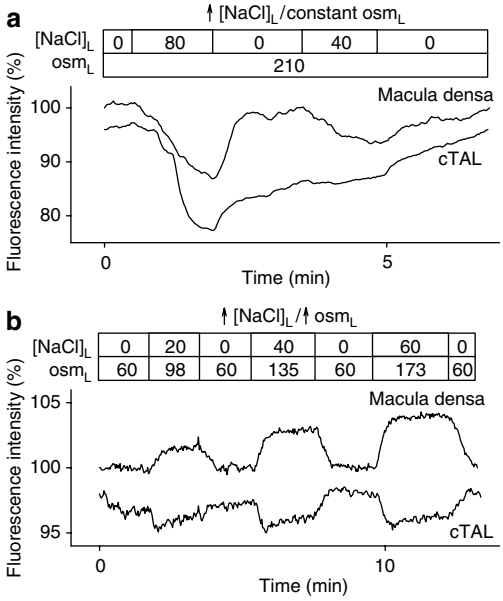

Figure 3 | $[NaCl]_L$-dependent changes in macula densa and cTAL cell volume. Representative tracings of calcein fluorescence intensity recorded from macula densa plaques and cTAL cells (cTAL) upon changes in $[NaCl]_L$ (shown in mmol/l above the tracings) (**a**) at constant osm_L (in mOsm/kg H_2O) and (**b**) with concomitant changes in osm_L. Cell volume changes in macula densa cells were sustained, and cTAL cells exhibited cell volume regulatory responses.

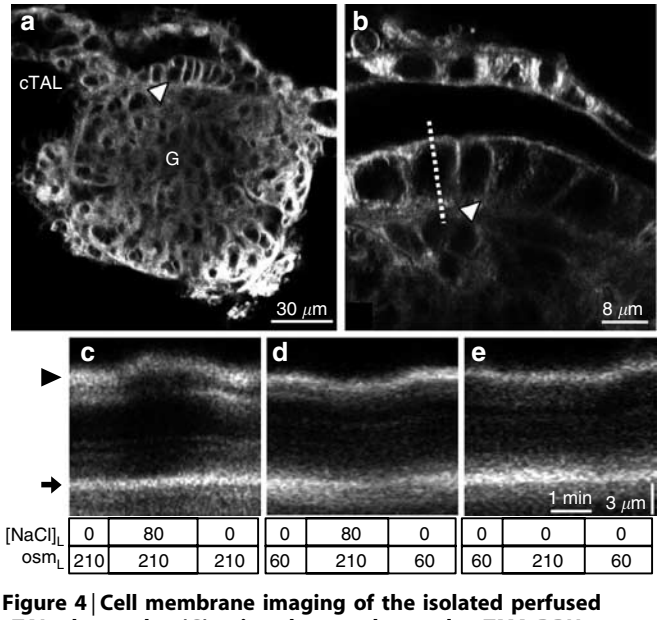

Figure 4 | Cell membrane imaging of the isolated perfused cTAL-glomerulus (G) using the membrane-dye TMA-DPH. Representative (**a**) low-power and (**b**) high-power multiphoton confocal fluorescence images of the preparation. White arrowhead denotes macula densa plaque. (**c**–**e**) Psedolinescan images generated from time-series images along the dotted line marked on (**b**) demonstrating the effects of alterations in $[NaCl]_L$ (in mmol/l; underneath the images) and/or osm_L (in mOsm/kg H_2O) on macula densa cell height. Black arrowhead and arrow denote apical and basolateral membrane of the macula densa cell, respectively. See Supplementary Material.

transient, suggesting active volume-regulatory mechanisms in cTAL cells (Figure 3b).

Effect of increases in $[NaCl]_L$ and osm_L on macula densa cell volume as assessed by cell membrane imaging

As determined by visualizing cell membranes with a membrane-staining dye 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) (Figure 4) and multiphoton excitation confocal microscopy, increases in $[NaCl]_L$ from 0 to 80 mmol/l at constant osm_L of 210 mOsm/kg H_2O produced reversible swelling of macula densa cells with an increase in cross-sectional area of $24 \pm 4\%$, whereas parallel increases in $[NaCl]_L$ and osm_L from 0 to 80 mmol/l and from 60 to 210 mOsm/kg H_2O, respectively, caused reversible shrinkage of macula densa cells (Figures 4c, d and 7 and Supplementary Material) with a decrease in cross-sectional area of $10 \pm 2\%$ (Figure 5). These changes in macula densa cross-sectional area were caused by alterations in macula densa cell height.

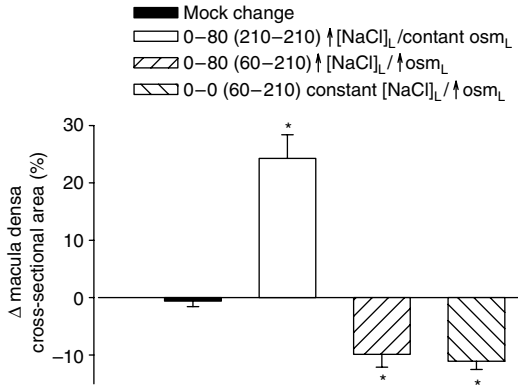

Figure 5 | $[NaCl]_L$- and osm_L-dependent changes in macula densa cross-sectional area. Effect of modulation of $[NaCl]_L$ (in mmol/l; see legend) and/or osm_L (in mOsm/kg H_2O; see legend in parentheses) on the macula densa cross-sectional area ($n = 6$; $^*P < 0.05$ as compared to values obtained with mock changes).

Effect of $[urea]_L$ on macula densa cell volume

Under normal physiological conditions, changes in osm_L occur predominantly either through alterations in $[NaCl]_L$ or $[urea]_L$. Studies in Figure 6 compare the changes in macula densa cell volume with addition of either NaCl or urea. As shown in Figure 6b, increases in $[urea]_L$ produced cell shrinkage, although at comparable osmotic concentration it was less effective than NaCl. Increases in $[urea]_L$ from 0 to 160 mmol/l (60–217 mOsm/kg H_2O) caused a reversible, dose-dependent increase in calcein fluorescence of $2.7 \pm 0.6\%$.

DISCUSSION

The present studies demonstrated that changes in cell volume in macula densa cells critically depend on the chosen experimental conditions. In spite of this finding, three major conclusions can be drawn from this work. First, increases in

$[NaCl]_L$ under physiological relevant conditions result in macula densa cell shrinkage. Second, consistent with previous observations[8,11] and in contrast to the adjacent cTAL cells, the apical membrane of macula densa cells is permeable to water. Third, macula densa cells lack effective cell volume regulatory mechanisms. As reported previously by others and also by our group,[12–14] increases in $[NaCl]_L$ at constant or close-to-constant osm_L ($\sim$300 mOsm/kg H_2O) produce macula densa cell swelling. This has been reconfirmed in our current studies using both the volume-sensitive dye calcein and also by visualizing cell membranes of macula densa cells with multiphoton fluorescence microscopy. Increases in $[NaCl]_L$ at constant osm_L also produced reversible decreases in calcein intensity in cTAL cells, suggesting cell swelling.

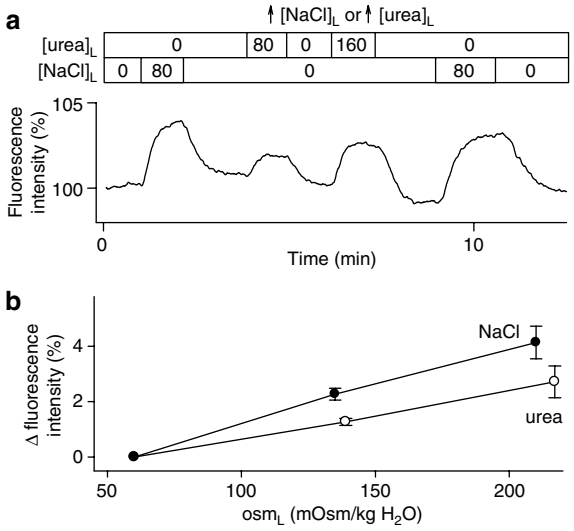

Figure 6 | Effect of $[urea]_L$ on macula densa cell volume. (**a**) Representative tracing of calcein fluorescence intensity recorded from macula densa cells demonstrating the effects of modulation of $[urea]_L$ or $[NaCl]_L$ (shown in mmol/l above the tracing) on calcein fluorescence with concomitant alterations in osm_L. (**b**) Effect of concomitant increases in $[urea]_L$ or $[NaCl]_L$ and osm_L (indicated in mOsm/kg H_2O) on macula densa calcein fluorescence intensity ($n = 5$).

In contrast, increasing $[NaCl]_L$ and osm_L concomitantly caused increases in calcein fluorescence, indicating shrinkage of macula densa cells. In the presence of concomitant changes in $[NaCl]_L$ and osm_L, there was a linear relationship between $[NaCl]_L$ and cell volume between 20 and 80 mmol/l $[NaCl]_L$ (Figure 2). Also, as shown in Figures 4 and 7 and in the Supplementary Movies S1 and S2, concomitant increases in $[NaCl]_L$ and osm_L led to modest decreases in cell height and a shrinkage of macula densa cells. Interestingly, in both calcein and membrane staining experiments, concomitant increases in $[NaCl]_L$ and osm_L produced reversible swelling of cTAL cells. Thus, these studies report a fundamental difference in the way macula densa and cTAL cells respond when both $[NaCl]_L$ and osm_L are altered concurrently.

We interpret these data as follows: it is firmly established that both macula densa and cTAL cells possess the apical Na^+:$2Cl^-$:K^+ cotransporter.[19] Thus, increases in $[NaCl]_L$ will result in uptake of NaCl into both cell types. Increasing intracellular [NaCl] would then result in a relative increase in the osmotic gradient for water entry into the cell. Presumably, both cTAL and macula densa cells possess a finite basolateral water permeability so that water can enter across the basolateral membranes. Studies by Gonzalez *et al.*[11] have shown that the osmotic water permeability of the macula densa basolateral membrane is about 13 times higher than that of the apical membrane. Thus, a steady-state increase in NaCl entry would lead to water uptake and cell swelling, which would be blocked by furosemide, an inhibitor of the Na^+:$2Cl^-$:K^+ cotransporter. Furosemide blocked the cell volume changes by 83%, a value that is in accordance with earlier studies indicating that $\sim$80% of apical Na^+ entry into the macula densa cells occurs via the Na^+:$2Cl^-$:K^+ cotransporter, whereas the Na^+:H^+ exchanger is responsible for the remainder.[20] However, the difference between macula densa and cTAL cells appears to be that whereas the apical membrane of cTAL cells is impermeable to water, macula densa cells exhibit finite water permeability. Thus, when both $[NaCl]_L$ and osm_L are increased, cTAL cells still swell, because there is no 'effective change' in the lumen to cell osmotic gradient in spite of

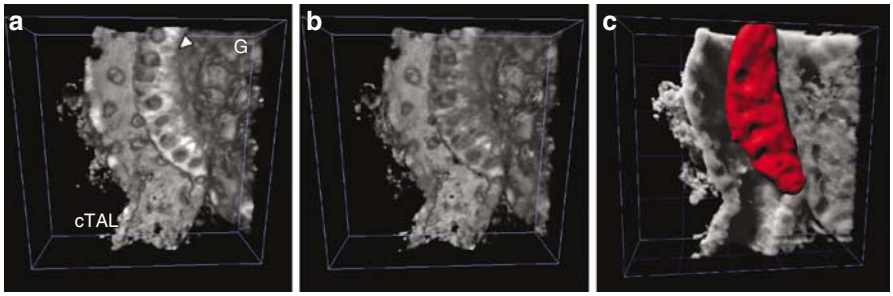

Figure 7 | Four-dimensional volume rendering and segmentation of the isolated perfused cTAL-glomerulus (G). (**a**, **b**) Representative volume-rendered images of the preparation visualized with cytosol-labeling dye $DiBAC_4(3)$ demonstrating the effect of concomitant reductions in $[NaCl]_L$ and osm_L from (**a**) 80 to (**b**) 0 mmol/l and from 210 to 60 mOsm/kg H_2O, respectively. White arrowhead denotes macula densa plaque. Note the swelling of macula densa cells. (**c**) Representative volume-rendered image of the same preparation merged with the intensity-segmented surface object of the macula densa plaque (red). As measured with isosurface tracking, macula densa cell volume increased from 28 152 to 29 510 μm^3 upon a decrease in $[NaCl]_L$ and osm_L from 80 to 0 mmol/l and from 210 to 60 mOsm/kg H_2O. Gridlines are 20 μm apart.

changes in osm_L, whereas in macula densa cells, the finite apical water permeability allows for an osmotic gradient to form across the apical membrane and, in the presence of elevated apical osmolality, the relative movement of water from cell to lumen. Although the present studies did not measure water permeability of macula densa cells, *per se*, the conclusion that can be reached is that the apical membrane of these cells is permeable enough to counteract the effects of enhanced NaCl entry. This is in accordance with earlier studies indicating that the water permeability of macula densa cells is ~12 times higher than that of the thick ascending limb.[11,21] Thus, the overall effect of increases in $[NaCl]_L$ (in the absence of mannitol or other osmotic agents) is to elicit cell shrinkage in macula densa cells. Interestingly, the fractional change in calcein fluorescence intensity (5.5%; Figure 2) was smaller than the observed change in macula densa cross-sectional area (10%; Figure 5). Although there might exist several explanations for this, one possible reason is that changes in macula densa cell volume do not necessarily parallel changes in macula densa cell height. An example of this is an earlier study[8] demonstrating concurrent heightening of macula densa cells with widening of the intercellular spaces.

These results suggest that there is a finite permeability for water across the apical membrane of macula densa cells. Physiologically, as flow is increased through the cTAL, the augmented flow would produce elevations in osm_L primarily owing to increases in $[NaCl]_L$ and $[urea]_L$. Effective changes in the osmotic gradient across cells are related to the osmotic reflection coefficient of a particular solute. Thus, in spite of the fact that NaCl is transported into macula densa cells, its osmotic reflection coefficient across the apical membrane of macula densa cells is presumably high as it is in most cell types.[22] This means that the osmotic driving force generated by Na^+ and Cl^- is almost equivalent to their concentration. However, despite some exceptions,[21] most cells have a finite permeability to urea and therefore a lower reflection coefficient. This is entirely consistent with the results obtained in the current studies, where an equal osmotic concentration of urea was less effective in causing cell shrinkage than NaCl.

One of the more interesting findings of the present studies is the lack of volume regulation in macula densa cells. Persson and his co-workers observed some signs of volume regulatory changes[11–13] in macula densa cells upon alterations in osmolality, but also reported that increases in $[NaCl]_L$ lead to sustained changes in cell volume.[11] Thus, there is conflicting information concerning the ability of macula densa cells to regulate cell volume. In response to cell swelling or shrinkage, most cells exhibit volume regulatory response. This is the case for cTAL cells (Figure 3b), where elevated $[NaCl]_L$ results in cell swelling (decrease in calcein fluorescence) followed by a time-dependent return toward control levels. In most cells, this regulatory volume decrease (RVD) is owing to the transport of osmotically active solutes from cell to extracellular fluid: chloride and potassium have both been shown to be involved in RVD in other cell types.[23]

However, macula densa cells (Figure 3b) show little tendency for volume regulation upon either cell shrinkage or cell swelling. One explanation for this is that previous work[20] indicated that intracellular $[Na^+]$ mirrored changes in $[NaCl]_L$ between 0 and 60 mmol/l. Thus, an elevation of $[NaCl]_L$ results in a sustained increase in intracellular $[Na^+]$. If this also applies to other intracellular electrolytes such as chloride and potassium, then it would explain why there is a lack of volume regulation in macula densa cells, that is, no volume regulatory influx or efflux of osmotically active solutes.

The finding that macula densa cells do not regulate volume is consistent with the role of this unique cell type as the sensor element for the TGF mechanism. In response to changes in the luminal environment, there are also sustained responses of basolateral membrane potential, intracellular $[Na^+]$, $[Ca^{2+}]$, and pH. Thus, a sustained response to alterations in the luminal environment appears to be a consistent characteristic of macula densa and may be an integral part of TGF signaling. However, a recent study failed to find a difference between the magnitude of *in vitro* afferent arteriole diameter responses to elevations in $[NaCl]_L$ whether the osm_L was maintained constant or was increased concomitantly,[24] suggesting that changes in macula densa cell volume might not be directly involved in the TGF signaling process. As this study was performed using very low (~11 mmol/l) and high (~81 mmol/l) $[NaCl]_L$, this finding does not eliminate the possibility that changes in cell volume can influence TGF responses over the physiological range of $[NaCl]_L$ and osm_L. On the other hand, it is now known that macula densa cells are involved in other paracrine or signaling processes. For instance, macula densa cells produce both nitric oxide[24,25] and prostaglandin E_2.[17] Thus, it is possible that changes in cell volume may affect the production of these signaling molecules or may alter other, as yet to be identified, function(s) of macula densa. It is also possible that cell volume may be involved in TGF resetting.[26] We speculate that macula densa cell volume changes might play a role in TGF resetting under conditions where luminal electrolyte or non-electrolyte delivery from the medulla is altered, such as during salt restriction or water deprivation. Finally, it is well known that changes in cell volume can produce a wide range of alterations in cell function, including changes in protein synthesis and matrix synthesis.[27–29] Therefore, it is possible that macula densa cell volume may influence not only the functional properties of macula densa cells, but also other elements within the juxtaglomerular apparatus.

MATERIALS AND METHODS

Materials

All materials were purchased from Sigma (St Louis, MO, USA) unless otherwise stated. Calcein/AM, TMA-DPH, and bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) were obtained from Molecular Probes Inc. (Eugene, OR, USA).

Tubule perfusion

The protocol used was approved by the Institutional Animal Care & Use Committee, University of Alabama at Birmingham, Birmingham, AL, USA. Individual cTAL segments containing the macula densa plaque with attached glomeruli were dissected from rabbit kidneys (New Zealand White rabbits; 0.5–1.0 kg; Myrtle's Rabbitry, Thompson Station, TN, USA; $n = 32$ animals in total) and perfused *in vitro* using methods similar to those described previously.[30] Dissection was performed at 4°C in an isosmotic, low-NaCl-containing Ringer dissection solution (Table 1). After transfer to a chamber that was mounted on the microscope, the tubule was cannulated and perfused with a perfusion solution. Tubules were bathed in a Ringer-like solution. All experiments were conducted at 37°C.

Experimental procedures

After having perfused with a control 'perfusion 0 NaCl' solution (Table 1) for 5 min, the perfusate was changed to 'perfusion 80 NaCl' solution for 5 min and then back to control. This procedure was performed at the beginning and at the end of each experiment, and the results of any test procedures performed between these periods were normalized to the average of these controls. To eliminate motion artifacts, a modified perfusion system was designed to provide for constant perfusion pressure during solution changes. Syringes supplying the perfusion solutions and the waste bottle were all pressurized. The perfusion flow is estimated to be ~30 nl/min.[8] Exchange of solutions was achieved by a constant gravity-driven flow within this pressurized system. As the solution flow was ~15 μl/s and the volume in the pipette head is ~3 μl, the time constant of perfusion exchange was well below the temporal resolution of the image acquisition.

Wide-field fluorescence microscopy

Macula densa cells were loaded with volume-sensing dye calcein by adding calcein/AM (10^{-5} mol/l), dissolved in dimethyl sulfoxide containing 15% w/v pluronic acid, to the luminal perfusate. Loading required ~5 min. Calcein fluorescence intensity was measured inside the macula densa cells with fluorescence microscopy (PTI, Lawrenceville, NJ, USA) using a Nikon S Fluor ×40 objective, a Nikon TE2000 microscope, and a cooled SenSys charge-coupled camera (Photometrics, Tucson, AZ, USA). Fluorescence was measured at an emission wavelength of 530 nm in response to an excitation wavelength of 495 nm. The spontaneous decline in fluorescence intensity was corrected for by linear curve-fitting and normalization.

Multiphoton fluorescence microscopy

In order to image plasma membranes to assess cell volume regulation, macula densa cells were stained for ~1 min with membrane-staining dye TMA-DPH (10^{-6} mol/l) in both the luminal perfusate and in the bath. TMA-DPH was excited at 800 nm using a diode-pumped, frequency-doubled Nd:vanadate pump laser (Verdi, 5 W) and a mode-locked titanium-sapphire femtosecond pulsed laser (Mira 900, both from Coherent, Santa Clara, CA, USA), coupled to a Leica DM IRBE microscope and Leica TCS SP confocal imaging system (Leica Microsystems, Heidelberg, Germany). The bandwidth at half-maximum intensity was ~7.4 nm. Fluorescence emission was detected using a Leica ×100 objective at 430 nm. In other experiments, the preparation was loaded with $DiBAC_4(3)$, a dye that labels the cytosol, and imaged in in xyzt dimensions with confocal microscopy at excitation and emission wavelengths of 488 and 530 nm, respectively. Volume rendering was performed with Voxx (Indiana Center for Biological Microscopy, Indianapolis, IN, USA) or Imaris (Bitplane, Zurich, Switzerland) software. Segmentation was performed based on intensity, supplemented with manual definition of the basolateral membrane. Tracking was performed using the connected components algorithm. Pseudolinescan images and annotated online movie were generated with ImageJ (National Institutes of Health, Bethesda, MD, USA) and Flash (Macromedia Inc., San Francisco, CA, USA) software, respectively.

Statistical analyses

Data are expressed as means ± s.e. Statistical analysis was performed with analysis of variance and Dunnett's or Bonferroni's test.

Table 1 | Composition of experimental solutions

	Dissection	Perfusion 0 NaCl[a] low osm	Perfusion 0 NaCl[a] high osm	Perfusion 80 NaCl	Perfusion 160 urea	Bath
NaCl	25	—	—	80	—	150
KCl	5	—	—	—	—	5
Na_2HPO_4	1.6	1.6	1.6	1.6	1.6	1.6
NaH_2PO_4	0.4	0.4	0.4	0.4	0.4	0.4
$CaCl_2$	1.5	—	—	—	—	1.5
$MgSO_4$	1	1	1	1	1	1
Glucose	5	5	5	5	5	5
K gluconate	—	5	5	5	5	—
Ca gluconate	—	3	3	3	3	—
NMDG cyclamate	125	—	—	—	—	—
Mannitol	—	—	145	—	—	—
Urea	—	—	—	—	160	—
HEPES	10	25	25	25	25	10
Osmolality (mOsm/kg H_2O)	325	60	210	210	217	305

Concentrations are given in mmol/l. The solutions used in the dose-response measurements, shown in Figures 2, 3, 6, and 7, were obtained by mixing 'perfusion 0 NaCl low osm' or 'perfusion 0 NaCl high osm' solutions with 'perfusion 80 NaCl' or 'perfusion 160 urea' solutions. The pH of the dissection and bathing solutions was set to 7.4 with NaOH, whereas that of the perfusion solutions was adjusted to 7.2 with NMDG at 37°C. Osmolality of solutions was set with mannitol using a freezing-point depression osmometer.

[a]The '0 NaCl' solution contains ~4 mmol/l of sodium but no chloride.

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SUPPLEMENTARY MATERIAL

Movie S1. Cell membrane imaging of the macula densa.

Movie S2. Four-dimensional imaging of macula densa cell volume changes.

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